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**Synthesis of a novel series of (Z)-3,5-disubstituted thiazolidine-2,4-diones as
promising anti-breast cancer agents**

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Abstract

A novel series of (*Z*)-3,5-disubstituted thiazolidine-2,4-diones **4-16** has been designed and synthesized. Preliminary screening of these compounds for their anti-breast cancer activity revealed that compounds **5**, **7**, and **9** possess the highest anti-cancer activities. The anti-tumor effects of compounds **5**, **7**, and **9** were evaluated against human breast cancer cell lines (MCF-7 and MDA-MB-231) and human breast cancer cells. They were also evaluated against normal non-cancerous breast cells, isolated from the same patients, to conclude about their use in a potential targeted therapy. Using MTT uptake method, these three compounds **5**, **7**, and **9** blunt the proliferation of these cancer cells in a dose-dependent manner with an IC₅₀ of 1.27, 1.50 and 1.31 μM respectively. Interestingly, using flow cytometry analysis these three compounds significantly mediated apoptosis of human breast cancer cells without affecting the survival of normal non-cancerous breast cells that were isolated from the same patients. Mechanistically, these compounds blunt the proliferation of MCF-7 breast cancer cells by robustly decreasing the phosphorylation of AKT, mTOR and the expression of VEGF and HIF-1α. Most importantly, compounds **5**, **7**, and **9** did not affect the phosphorylation and expression of these crucial cellular factors in normal non-cancerous breast cells that were isolated from the same patients. Additionally, using Western blot analysis the three compounds significantly ($P < 0.05$) decreased the expression of the anti-apoptotic Bcl-2 members (Bcl-2, Bcl-XL and Mcl-1) and increased the expression of the pro-apoptotic Bcl-2 members (Bak, Bax and Bim) in MCF-7, MDA-MB-231 and human breast cancer cells making these breast cancer cells susceptible for apoptosis induction. Taken together, these data provide great evidences for the inhibitory activity of these compounds against breast cancer cells without affecting the normal breast cells.

Key Words: Thiazolidinediones; thiazolidine-2,4-diones; apoptosis; Bcl-2 family; anti-breast cancer.

1. Introduction

Cancer, which affected about 18.1 million new cases in 2018, is the second leading cause of death after the heart diseases across the world [1]. Among the various types of malignant tumors reported so far, breast cancer is the most frequent cancer in women and represents the second most prominent reason for deaths among women [2, 3]. Despite the big efforts devoted to fight against different types of cancer, yet this disease remains one of the major concerns worldwide. Therefore, the discovery of new and potent anticancer drugs is always in demand.

Thiazolidine-2,4-diones (TZDs), represent a very important class of heterocyclic compounds which is highly explored in the literature. TZDs possess a wide spectrum of biological activities such as antidiabetic [4-9], anti-infectious [10-15], anti-inflammatory [16, 17], analgesic [18], antioxidant [19], immunomodulatory [20], overactive bladder inhibitory [21] and insecticide [22] activities. TZDs also possess inhibitory activity against tyrosinase [23], hyperlipidimia [24] and acute liver injury [25].

Furthermore, it was reported [26] that TZDs affected cancer development, progression and metastasis, among which the Raf/MEK/extracellular signal regulated kinase (ERK) [27], phosphatidylinositol 3-kinase (PI3K)/AKT [28], Wnt signal transduction pathways [29], PIM-1 and PIM-2 protein kinases overexpression [30], androgen receptor stimulated genes [31] and peroxisome proliferator-activated receptors (PPARs) signaling cascades [32] which are the most commonly up-regulated in human cancers. Most importantly, two recent reports demonstrated anti-breast cancer activity of TZDs, but the molecular mechanisms underlying the treatment of breast cancer cells with these compounds were not clarified [31,33]. Thus, in our present study three synthesized TZDs (**5**, **7** and **9**) were

evaluated against human breast cancer cell lines (MCF-7 and MDA-MB-231) and human breast cancer cells. They were also evaluated against human normal non-cancerous breast cells which were isolated from the same breast cancer patients. We also monitored the molecular mechanisms underlying treatment of breast cancer cells with the three thiazolidinediones **5**, **7** and **9**.

2. Results and discussion

2.1. Chemistry

In the present study, some novel thiazolidine-2,4-dione (TZD) derivatives were synthesized using thiazolidine-2,4-dione (**1**) [34] as a starting material. This latter compound has the advantage of possessing two reactive centers: the active methylene group at position 5 and the nucleophilic nitrogen anion at position 3, of its potassium salt, which together allow the synthesis of a variety of TZD derivatives. Accordingly, the active methylene group of compound **1** was reacted with 3,4,5-trimethoxybenzaldehyde *via* Knoevenagel reaction to give the corresponding (*Z*)-5-(3,4,5-trimethoxybenzylidene)thiazolidine-2,4-dione isomer (**2**). This reaction was carried out in boiling anhydrous toluene in the presence of few drops of piperidine and glacial acetic acid using a Dean-Stark apparatus. The idea behind using the 3,4,5-trimethoxybenzaldehyde, in the above condensation reaction, was to mimic the 3,4,5-trimethoxybenzene moiety found in many natural anticancer drugs described in the literature such as combretastatin A-4 (Figure I) [35] or colchicine (Figure II) [36].

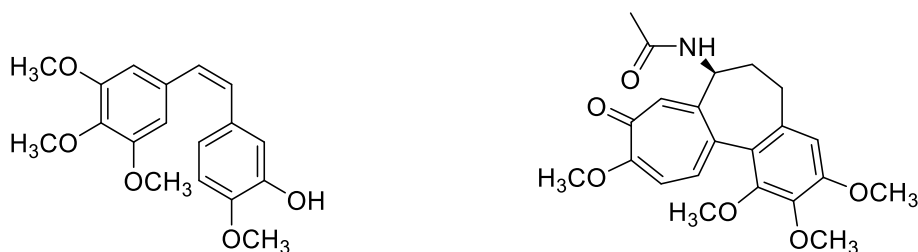
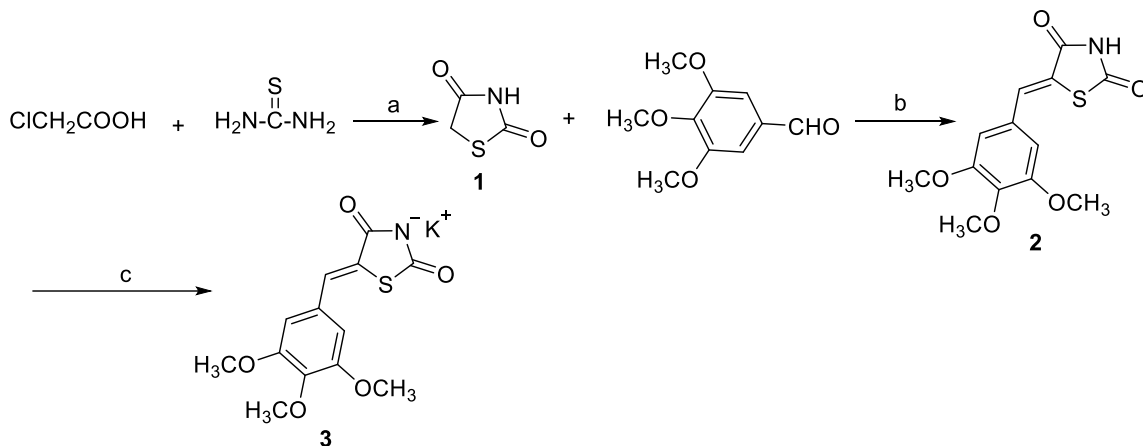


Figure 1. Combretastatin A-4**Figure 2.** Colchicine

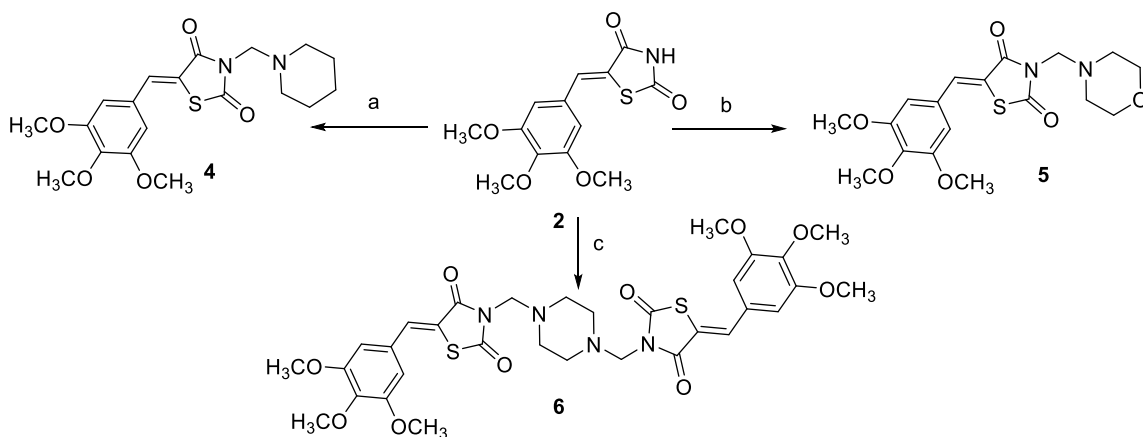
Compound **2** was then converted into its potassium salt **3** by treatment with an ethanolic KOH solution (Scheme 1).



Reagents and conditions: (a) i. water, 0-5°C, 20 min, ii. HCl, reflux, 12 h, (b) anhydrous toluene, drops of piperidine and CH₃COOH, reflux, 15 h. (c) KOH/ethanol, 80 °C, 20 min.

Scheme 1. General synthesis of compound **3**.

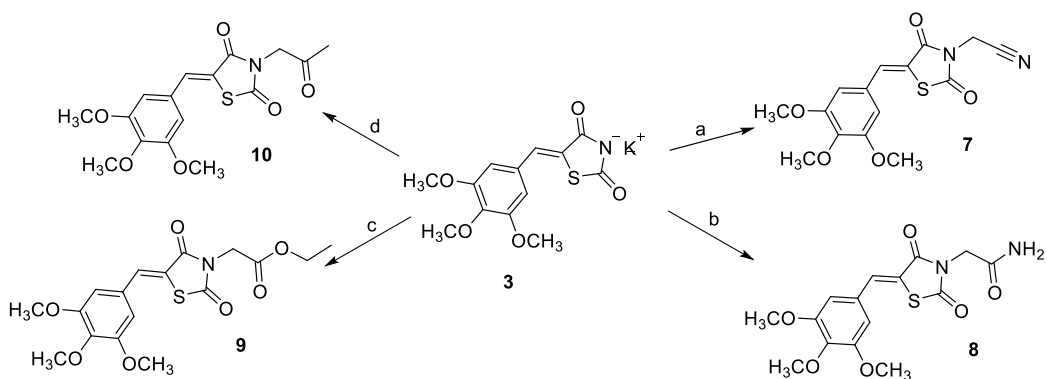
Both compounds **2** and **3** were used as synthons in the synthesis of the title compounds. Thus, when Mannich reaction was applied to compound **2** using piperidine, morpholine or piperazine as a secondary amine, the corresponding thiazolidinedione derivatives **4-6** were obtained respectively (Scheme 2).



Reagents and conditions: (a) formaldehyde, piperidine, ethanol, reflux, 6h. (b) formaldehyde, morpholine, ethanol, reflux, 6h. (c) formaldehyde, piperazine, ethanol, reflux, 6h.

Scheme 2. General synthesis of compound 4-6.

Alternatively, other N-substituted TZD derivatives **7-16** could be obtained *via* the reaction of the salt **3** with halogeno- and dihalogeno reagents. Thus, when **3** was reacted with some chloro reagents, such as chloroacetonitrile, 2-chloroacetamide, ethyl chloroacetate, and chloroacetone in boiling DMF, the corresponding (*Z*)-N-substituted 5-(3,4,5-trimethoxybenzylidene)thiazolidinediones **7-10** were obtained (Scheme 3).



Reagents and conditions: (a) chloroacetonitrile, DMF, reflux, 6h. (b) 2-chloroacetamide, DMF, reflux, 6h. (c) ethyl chloroacetate, DMF, reflux, 6h. (d) chloroacetone, DMF, reflux, 6h.

Scheme 3. General synthesis of compounds 7-10.

The $^1\text{H-NMR}$ spectra of the basic structure of compounds **4-10** showed characteristic signals of the 9 protons of the three methoxy groups (in addition to those of the other substituents at the N atom). In deuterated chloroform, these 9 protons appeared as a singlet at δ 3.92 ppm as in compounds **4**, **6** and **7** (Figure 3A). However, in deuterated DMSO, they appeared as two singlets at δ 3.70-3.74 and 3.84-3.85 ppm, integrated for 3 protons (of the 4-methoxy substituent) and 6 protons (of the 3,5-dimethoxy substituents) as in compounds **5**, **8-10** respectively (Figure 3B). The exocyclic methylene group of compounds **4-10** appeared as a singlet at δ 4.24-4.73 ppm (H_3 , Figures 3A and 3B). The two aromatic protons appeared as a singlet at δ 6.75-6.98 ppm (H_1 , Figures 3A and 3B).

The methyldene proton $-\text{CH}=\text{}$ appeared downfield as a singlet at 7.80-7.95 ppm (H_2 , Figures 3A and 3B), deshielded by the anisotropic effect exerted by the nearby carbonyl group, proving a *Z*-configuration [19, 37]. No signal indicating the formation of the *E*-configuration was observed, since the chemical shift of the $-\text{CH}=\text{}$ proton in this case should appear around 6.25 ppm [19]. The unambiguous exclusive formation of *Z*-configuration of these products was confirmed by X-ray analysis as was shown in our X-ray report [38]. The ^{13}C -NMR spectra are consistent with the structure of all the compounds obtained.

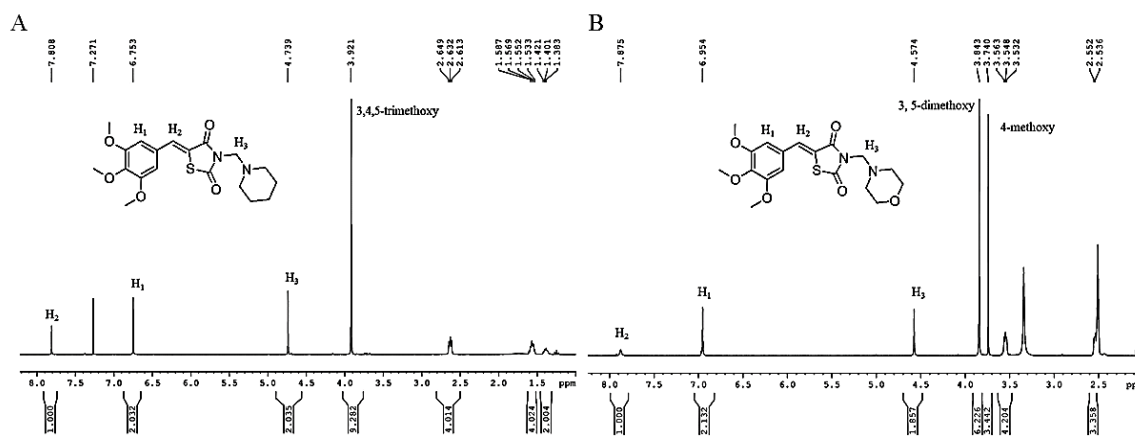
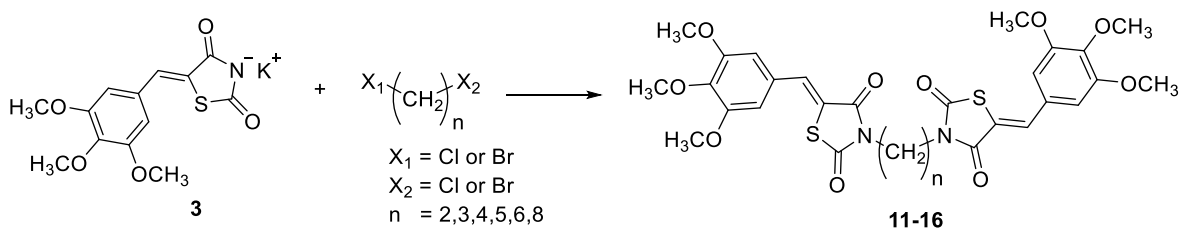


Figure 3. ^1H NMR spectra (A) and (B) of compound **4** and **5**, respectively.

On the other hand, a series of (*Z*)-bis(5-(3,4,5-trimethoxybenzylidene)thiazolidin-3-yl)alkanes **11-16** with alkane spacers was obtained through the reaction of **3** with dihalogeno alkanes in DMF under reflux (Scheme 4). All analyses of **11-16** are consistent with the structures obtained.



Reagents and conditions: dihalogeno alkanes, DMF, reflux, 6 hr.

Scheme 4. General synthesis of compound **11-16**.

2.2. Biology

The treatment of drug-resistant cancer is a clinical challenge, and thus searching for novel anticancer drugs is critically important [39]. Therefore, an intense research effort is underway to reveal the underlying mechanisms of apoptosis and cancer progression [40]. Clinically, many chemotherapeutic drugs provide a satisfactory response upon their first exposure to the tumors, but they cause a variety of side effects to the patients. Therefore, there is an urgent need for potent and selective anticancer drugs [41]. We focused our attention in the present study to investigate the anti-tumor potential of the best TZD derivatives and to clarify the underlying molecular mechanisms in the human breast carcinoma cell lines MDA-MB-231 and MCF-7. We also monitored whether these analogues exert cytotoxic effects on both human breast carcinoma cell and non-tumorigenic normal breast cell. There are several mechanisms of anticancer activity of thiazolidine-2,4-dione derivatives already discussed in the literature. The most known ones are induction of apoptosis, cell differentiation, and cell cycle arrest [42]. Proliferation is a crucial process in the maintenance and progression of cancer cells. The ability of cancer cells to escape from apoptosis and continue to proliferate is one of the fundamental hallmarks of cancer and is a major target for cancer treatment [39]. In the present study, we found that compounds **5**, **7** and **9** inhibit the proliferation and cell viability of human breast cancer cells.

2.2.1. Compounds 5, 7 and 9 blunt the proliferation and cell viability of human breast cancer cells

Preliminary anti-proliferative screening of all the compounds prepared has been performed against MCF-7 cell line using MTT uptake method to identify the most potent compounds.

The best results were shown by the thiazolidinediones **5**, **7** and **9** (Table 1, Figure 4).

Human breast cancer cells were isolated from breast cancer patients who were admitted to South Egypt Cancer Institute, Assiut University, Egypt. The isolated breast cancer cells were then treated with different concentrations of the synthesized compounds **4-16** for 12h and their anti-tumor effect was monitored using MTT uptake method. Three compounds, **5**, **7** and **9** showed a dose dependent anti-proliferative effect with an IC₅₀ of 1.27, 1.50 and 1.31 μ M respectively (**Figure 4**). These experiments were performed in triplicate and the results were expressed as the mean percentage of viable cells \pm SEM. These results were confirmed by a Trypan blue exclusion test, which was used to determine cell viability and numerical cell counts simultaneously in all experiments.

2.2.2. Compounds 5, 7 and 9 robustly mediate apoptosis of human breast cancer cells.

The inhibition of cancer cell proliferation, the cessation of cell cycle progression and the induction of apoptosis have all been targeted in chemotherapeutic strategies for the treatment of various types of cancer. Given the anti-proliferative activity of compounds **5**, **7** and **9** against human breast cancer cells, we next evaluated their impact on the induction of apoptosis in human normal non-cancerous breast cells and in human breast cancer cells, that were isolated from the same patients, using propidium iodide (PI)/Annexin V double staining, followed by flow cytometry analysis. Both human breast cancer- and normal cells were treated with 1.27, 1.50 and 1.31 μ M of compounds **5**, **7** and **9** respectively for 12 h. The data from a representative experiment are presented in the dot plot shown, and the

percentages of viable cells (lower left quadrant), early apoptotic cells (lower right quadrant) and late apoptotic cells (upper right quadrant) were determined (**Figure 2-A- 6 A**). In the human normal non-cancerous breast cells, the percentages of apoptotic cells (early and late apoptotic) were 5% in untreated cells, 5%, 4.5% and 8%, in **5**-, **7**-, and **9**-treated cells, respectively. However, in human breast cancer cells the percentages of apoptotic cells (early and late apoptotic) were 12.5% in untreated cells, 65.5%, 77%, and 69%, in **5**-, **7**-, and **9**-treated cells, respectively. Pooled data from three different experiments are expressed as the mean percentage of apoptotic cells \pm SEM revealed that compounds **5**, **7** and **9** significantly ($P < 0.05$) induced apoptosis of human breast cancer cells without affecting the normal non-cancerous breast cells. The effect of compounds **7** (**Figure 2-B 6 B**, closed black bars), **9** (**Figure 2-C 6 C**, closed gray bars) and **5** (**Figure 2-D 6 D**, hatched bars) on the induction of apoptosis of human breast cancer cells were significantly obvious.

2.2.3. Treatment with compounds 5, 7 and 9 induces apoptosis in human breast cancer cells through direct effect on PI3K/AKT/mTOR, VEGF and HIF-1 α signaling

The mechanism of induced apoptosis upon treatment with compounds **5**, **7** and **9** was investigated by treating MCF-7 cell lines with 1.27, 1.50 and 1.31 μ M of **5**, **7** and **9** respectively for 12 h, followed by lysing the cells in lysis buffer. Western blot analysis was then used to analyze the phosphorylation of AKT and mTOR, and the expression of VEGF and HIF-1 α . The results of a representative experiment (out of five) on MCF-7 breast cancer cells showed that the treatment with compounds **5**, **7** and **9** markedly decreased the phosphorylation of AKT (**Figure 3-A 7 A**) and mTOR (**Figure 3-C 7 C**), and also decreased the expression levels of VEGF and HIF-1 α (**Figure 3-E 7E**) compared with the

medium (0)-treated cells. The phosphorylation levels of AKT and mTOR were normalized to the total relevant proteins (T-AKT and T-mTOR) and expression levels of VEGF and HIF-1 α were normalized to total β -actin protein levels. The data from 5 independent experiments performed on MCF-7 cells showed that the treatment with compounds **5**, **7** and **9** significantly ($P < 0.05$) inhibited the phosphorylation of AKT (**Figure 3-B 7B**) and mTOR (**Figure 3-D 7D**) and the expression levels of VEGF and HIF-1 α (**Figure 3-F 7F**) compared with medium (0)-treated cells. This experiment was then repeated using both human normal non-cancerous breast cells and human breast cancer cells which were isolated from the same breast cancer patients.

The results of a representative experiment (out of five) showed that the phosphorylation of AKT (**Figure 4-A 8A**) and mTOR (**Figure 4-C 8C**), and the expression of VEGF and HIF-1 α (**Figure 4-E 8E**) were obviously increased in human breast cancer cells in comparison with human non-cancerous breast cells. Additionally, treatment of human breast cancer cells with compounds **5**, **7** and **9** markedly decreased the phosphorylation of AKT (**Figure 4-A 8A**) and mTOR (**Figure 4-C 8C**) and also decreased the expression levels of VEGF and HIF-1 α (**Figure 4-E 8E**) compared with the medium (0)-treated cells. Interestingly, no obvious effects were observed with the tested compounds on human non-cancerous breast cells. As previously, after normalization of the data obtained from five independent experiments, the results clearly indicated that the treatment with compounds **5**, **7** and **9** significantly ($P < 0.05$) inhibited the phosphorylation of AKT (**Figure 4-B 8B**) and mTOR (**Figure 4-D 8D**) and the expression levels of VEGF and HIF-1 α (**Figure 4-F 8F**) compared with medium (0)-treated cells.

2.2.4. Treatment with compounds 5, 7 and 9 induces apoptosis in human breast cancer cells through direct effects on the Bcl-2 family members.

We have investigated the expression levels of Bcl-2 family members (including the anti-apoptotic members Bcl-2, Bcl-XL and Mcl-1, and the pro-apoptotic members Bak, Bax and Bim) after treatment with 1.27, 1.50 and 1.31 μ M of these three compounds **5**, **7** and **9** respectively. The results of a representative experiment (out of five) on MCF-7 cell line demonstrated that treatment with compounds **5**, **7** and **9** markedly decreased the expression levels of the anti-apoptotic Bcl-2 members Bcl-2, Bcl-XL and Mcl-1 (**Figure 5-A 9A**) and clearly increased the expression levels of the pro-apoptotic Bcl-2 members Bak, Bax and Bim (**Figure 5-B 9B**) compared with the medium (0)-treated cells. The expression levels of the analyzed proteins were normalized to total GAPDH protein levels.-These results were significant ($P < 0.05$), as shown in Figures 9C and 9D, and revealed that the decreased expression of the anti-apoptotic Bcl-2 members was accompanied by an increase in the expression of the pro-apoptotic Bcl-2 members, which favors apoptosis induction. Similar results were obtained using MDA-MB-231 human breast cancer cell line (**Figure 6- 10**).

This experiment was then repeated using both human normal non-cancerous breast cells and human breast cancer cells that were isolated from the same patients. The results of a representative experiment (out of five) clearly illustrated that the expression levels of the anti-apoptotic Bcl-2 members Bcl-2, Bcl-XL and Mcl-1 (**Figure 7-A 11A**) were clearly up-regulated, while the expression levels of the pro-apoptotic Bcl-2 members Bak, Bax and Bim (**Figure 7-B 11B**) were clearly down-regulated in the human breast cancer cells as compared with the expression levels in the human normal non-cancerous breast cells. Additionally, treatment of human breast cancer cells with compounds **5**, **7** and **9** markedly

decreased the expression levels of the anti-apoptotic Bcl-2 members Bcl-2, Bcl-xL and Mcl-1 (**Figure 7A 11A**) and clearly increased the expression levels of the pro-apoptotic Bcl-2 members Bak, Bax and Bim (**Figure 7B 11B**) compared with the medium (0)-treated cells. Normalized results allowed considering the impact of compounds **5**, **7** and **9** on the repression of the anti-apoptotic Bcl-2 members and the induction of the pro-apoptotic proteins (**Figures 7C 11C** and **7D 11D**).

Interestingly, the modulatory effects of compounds **5**, **7** and **9** on the expression of Bcl-2 family members were restricted only on the human breast cancer cells without affecting the human normal non-cancerous breast cells.

3. Conclusion

A new series of thiazolidine-2,4-diones incorporating the 5-(3,4,5-trimethoxybenzylidene) moiety, found in some natural anticancer drugs, has been designed and synthesized. The synthesized TZDs were tested for their anti-breast cancer activity against human cancer cell lines (MCF-7 and MDA-MB-231) and human breast cancer cells and also against normal non-cancerous breast cells that were isolated from the same patients. Compounds **5**, **7** and **9** were shown to be the most active derivatives against breast cancer by evaluating their activities using MTT assay, flow cytometry and Western blot analysis. Compounds **5**, **7** and **9** inhibit the proliferation of breast cancer cells in a dose dependent manner with an IC₅₀ of 1.27, 1.50 and 1.31 μ M respectively. Interestingly, they induced apoptosis of human breast cancer cells without affecting the normal non-cancerous breast cells. They mediated apoptosis in MCF-7, MDA-MB-231 and human breast cancer cells through decreasing the expression of the anti-apoptotic Bcl-2 members (Bcl-2, Bcl-xL and Mcl-1) and increasing the expression of the pro-apoptotic Bcl-2 members (Bak, Bax and Bim).

Furthermore, the synthesized three compounds decreased the phosphorylation of AKT and mTOR and also decreased the expression levels of VEGF and HIF-1 α . Further experiments will be performed in due course in order to elucidate their molecular mechanisms and identify their potential therapeutic targets.

4. Experimental section

4.1. Chemistry

4.1.1. General methods

All melting points were determined on a Stuart melting point apparatus SMP3 and are uncorrected. IR spectra were recorded on a Nicolet iS10 FT-IR spectrometer using KBr wafer technique. The ^1H -NMR spectra were recorded on a Bruker Avance 300 spectrometer operating at 300 MHz (^1H) or 75 MHz (^{13}C). ^1H - and ^{13}C -NMR chemical shifts were reported in parts per million (ppm) and were referenced to the residual proton peaks in deuterated solvents; CDCl_3 (7.26 ppm for ^1H and 76.90 ppm for ^{13}C) and $\text{DMSO-}d_6$ (2.50 ppm for ^1H and 39.70 ppm for ^{13}C). Multiplicities are represented by s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Coupling constants (J) are reported in Hertz (Hz). Mass spectra were recorded with an LCMS (Waters Alliance Micromass ZQ 2000). LCMS analysis was performed using a Waters XBridge C18 column (5 μm particle size column, dimensions 50 mm x 4.6 mm). A gradient starting from 98% H_2O /formate buffer 5 mM (pH 3.8) and reaching 100% CH_3CN / formate buffer 5 mM (pH 3.8) within 4 min at a flow rate of 2 ml/min was used followed by a return to the starting conditions within 1 min. Elemental analyses (C, H, N) were carried out using a Perkin Elmer 240 C Micro analyzer and they were found in a good agreement with the theoretical values within $\pm 0.4\%$. Thiazolidine-2,4-dione (**1**) was prepared according a literature procedure [34].

4.1.2. (Z)-5-(3,4,5-Trimethoxybenzylidene)thiazolidine-2,4-dione (2)

A mixture of equimolar amounts (20 mmol) of thiazolidine-2,4-dione (**1**) (2.34 g) and 3,4,5-trimethoxybenzaldehyde (3.92 g) in anhydrous toluene (50 ml) was heated under reflux in the presence of few drops of acetic acid and piperidine in a flask equipped with a Dean-Stark apparatus. The reaction mixture was heated under reflux for 15 h. After cooling, the solid product obtained was filtered, washed with little toluene and dried. Recrystallization from cyclohexane gave light yellow crystals, yield 5.49 g (93%), mp 185-187 °C. IR (ν cm^{-1}): 3176 (NH), 3020 (CH arom.), 2992 (CH aliph.), 2950 (CH aliph.), 2836 (CH aliph.), 1748 (C=O), 1698 (C=O), 1606, 1576, 1506, and 1451 (C=C arom.). ^1H -NMR (300 MHz, DMSO- d_6): δ 3.72 (s, 3H, -OCH₃), 3.82 (s, 6H, 2 -OCH₃), 6.91 (s, 2H, Ar-H), 7.25 (s, 1H, -CH=), 12.59 (s, 1H, NH); ^{13}C NMR (75 MHz, DMSO- d_6): δ 56.4 (2 -OCH₃), 60.6 (OCH₃), 107.9 (2 CH), 122.9 (C), 128.9 (C), 132.5 (C), 139.8 (CH), 153.6 (2C), 167.7 (C=O), 168.3 (C=O). MS (ESI) m/z 294.3 [M-H]⁻. Anal. calcd. for C₁₃H₁₃NO₅S (295.31): C, 52.87; H, 4.44; N, 4.74. Found: C, 52.90; H, 4.19; N, 4.66 %.

4.1.3. (Z)-5-(3,4,5-Trimethoxybenzylidene)thiazolidine-2,4-dione potassium salt (3)

A solution of potassium hydroxide (0.062 g, 1.1 mmol) in ethanol (3 ml) was added to a hot solution of compound **2** (0.3 g, 1 mmol) in ethanol (3 ml). The reaction mixture was then heated with stirring at 80 °C for 20 min. The white fluffy long needles obtained were filtered, washed with ethanol and dried. Yield (0.28 g (83%)), mp 250-255 °C (decomp.). IR (ν cm^{-1}): 3026 (CH arom.), 2955 (CH aliph.), 2849 (CH aliph.), 1742 (C=O), 1690 (C=O), 1607, 1577, 1507, 1577 and 1454 (C=C arom.). ^1H -NMR (300 MHz, DMSO- d_6) δ (ppm): 3.68 (s, 3H, -OCH₃), 3.80 (s, 6H, 2 -OCH₃), 6.86 (s, 2H, ArH), 7.25 (s, 1H, =CH-). ^{13}C -NMR (75 MHz, DMSO- d_6) δ (ppm): 56.2 (2 OCH₃), 60.5 (OCH₃), 106.8 (2 CH),

122.8 (C), 132.0 (C), 136.0 (C), 137.7 (CH), 153.3 (2 C), 176.0 (C=O), 183.3 (C=O). MS (ESI⁻) m/z 294.3 [M-H]⁻. Anal. calcd. for C₁₃H₁₂KNO₅S (333.40): C, 46.83; H, 3.63; N, 4.20. Found: C, 46.58; H, 3.50; N, 4.00 %.

4.1.4. General procedure for the synthesis of compounds 4-6.

To a mixture of piperidine (0.1 ml, 1 mmol), morpholine (0.087 ml, 1 mmol) or piperazine (0.08 ml, 1 mmol) and formaldehyde (0.5 ml, 13.5 mmol) in methanol (5 ml), compound **2** (0.3 g, 1 mmol) was added. The reaction mixture was stirred under reflux for 6 hr in the presence of few drops of acetic acid. After cooling the solid product obtained was filtered, washed with water and dried. Recrystallization from ethanol gave crystals.

4.1.4.1. (Z)-3-(Piperidin-1-ylmethyl)-5-(3,4,5-trimethoxybenzylidene)thiazolidine-2,4-dione (**4**)

White crystals, yield 0.26 g (65%), mp 147-149 °C. **IR** (ν cm⁻¹): 3008 (CH arom.), 2921 (CH aliph.), 2833 (CH aliph.), 1739 (C=O), 1682 (C=O), 1605, 1574, 1501 and 1452 (C=C arom.). **¹H-NMR** (300 MHz, CDCl₃) δ (ppm): 1.40 (m, 2H, CH₂), 1.55 (m, 4H, 2 CH₂), 2.60 (t, 4H, *J* = 5.4 Hz, 2 N-CH₂), 3.92 (s, 9H, 3 O-CH₃), 4.73 (s, 2H, CH₂), 6.75 (s, 2H, ArH), 7.80 (s, 1H, =CH-). **¹³C-NMR** (75 MHz, CDCl₃) δ (ppm): 23.7 (CH₂), 25.9 (2 CH₂), 51.9 (2 -NCH₂), 56.2 (2 -OCH₃), 61.0 (-OCH₃), 64.5 (-NCH₂N-), 107.5 (2 CH), 120.7 (C), 128.8 (C), 133.7 (C), 140.2 (CH), 153.6 (2 C), 167.6 (C=O), 168.9 (C=O). MS (ESI⁺) m/z 296.0 [M+H-CH₂-piperidine]⁺. Anal. calcd. for C₁₉H₂₄N₂O₅S (392.47): C, 58.15; H, 6.16; N, 7.14. Found: C, 58.00; H, 6.26; N, 6.99 %.

4.1.4.2. (Z)-3-(Morpholin-4-ylmethyl)-5-(3,4,5-trimethoxybenzylidene)thiazolidine-2,4-dione (**5**)

Yellow crystals, yield 0.36 g (90%), mp 143-145 °C. **IR** (ν cm^{-1}): 3017 (CH arom.), 2952 (CH aliph.), 2854 (CH aliph.), 1740 (C=O), 1682 (C=O), 1604, 1575, 1501 and 1449 (C=C arom.). **¹H-NMR** (300 MHz, DMSO-*d*₆) δ (ppm): 2.53 (t, 4H, *J* = 4.5 Hz, 2 -NCH₂), 3.55 (t, 4H, *J* = 4.5 Hz, 2 -OCH₂), 3.74 (s, 3H, -OCH₃), 3.84 (s, 6H, 2 -OCH₃), 4.57 (s, 2H, CH₂), 6.95 (s, 2H, ArH), 7.87 (s, 1H, =CH-). **¹³C-NMR** (75 MHz, DMSO-*d*₆) δ (ppm): 50.9 (2 CH₂), 56.5 (2 -OCH₃), 60.7 (-OCH₃), 63.7 (-N-CH₂-N-), 66.5 (2 CH₂), 108.0 (2 CH), 120.9 (C), 129.0 (C), 133.7 (C), 140.0 (CH), 153.7 (2 C), 167.2 (C=O), 168.9 (C=O). **MS** (ESI⁺) *m/z* 296.0 [M+H-CH₂-morpholine]⁺. Anal. calcd. for C₁₈H₂₂N₂O₆S (394.44): C, 54.81; H, 5.62; N, 7.10. Found: C, 54.60; H, 5.80; N, 6.84 %.

4.1.4.3. 3,3'-(Piperazine-1,4-diylbis(methylene))bis(5-((Z)-3,4,5-trimethoxybenzylidene)thiazolidine-2,4-dione) (6)

Yellow crystals, yield 0.60 g (84%), mp 205-207 °C. **IR** (ν cm^{-1}): 3000 (CH arom.), 2992 (CH aliph.), 2944 (CH aliph.), 2828 (CH aliph.), 1737 (C=O), 1678 (C=O), 1603, 1576, 1504 and 1466 (C=C arom.). **¹H-NMR** (300 MHz, CDCl₃) δ (ppm): 2.70 (s, 8H, piperazine), 3.92 (s, 6H, 2 -OCH₃), 3.93 (s, 12H, 4 -OCH₃), 4.71 (s, 4H, 2 -NCH₂N-), 6.74 (s, 4H, ArH), 7.81 (s, 2H, 2 -CH=). **¹³C-NMR** (75 MHz, CDCl₃) δ (ppm): 50.4 (4CH₂), 56.2 (4 OCH₃), 61.0 (2 OCH₃), 63.1 (2CH₂), 107.5 (4C), 120.3 (2C), 128.6 (2C), 134.1 (2C), 140.3 (2CH), 153.6 (4C), 167.3 (C=O), 168.8 (C=O). **MS** (ESI⁺) *m/z* 296.0 [M+H-H₂-substituted piperazine]⁺. Anal. calcd. for C₃₂H₃₆N₄O₁₀S₂ (700.78): C, 54.85; H, 5.18; N, 8.00. Found: C, 54.66; H, 5.30; N, 7.89 %.

4.1.5. General procedure for the synthesis of compounds 7-10.

A mixture of the potassium salt **3** (0.33 g, 1 mmol) and chloroacetonitrile (0.082 g, 0.07 ml, 1.1 mmol), chloroacetamide (0.1 g, 1.1 mmol), ethyl chloroacetate (0.135 g, 0.116 ml,

1.1 mmol), chloroacetone (0.1 g, 0.09 ml, 1.1 mmol) in DMF (5 ml) was heated under reflux for 6 hr. After cooling the solid product obtained was filtered, washed with water, dried and recrystallized.

4.1.5.1. ((Z)-5-(3,4,5-Trimethoxybenzylidene)thiazolidine-2,4-dione-3-yl)acetonitrile (7)

Yellow needles (recrystallization from ethanol), yield 0.26 g (87%), mp 173-175 °C. **IR** (ν cm^{-1}): 3026 (CH arom.), 2998 (CH aliph.), 2836 (CH aliph.), 2222 (CN), 1732 (C=O), 1681 (C=O), 1608, 1573 and 1448 (C=C arom.). **$^1\text{H-NMR}$** (300 MHz, CDCl_3) δ (ppm): 3.93 (s, 9H, 3 -OCH₃), 4.63 (s, 2H, CH₂), 6.75 (s, 2H, ArH), 7.92 (s, 1H, =CH-). **$^{13}\text{C-NMR}$** (75 MHz, CDCl_3) δ (ppm): 28.2 (CH₂), 56.3 (2 -OCH₃), 61.1 (-OCH₃), 109.5 (2 CH), 112.8 (CN), 118.5 (C), 127.9 (C), 136.3 (C), 141.0 (CH), 153.7 (2 C), 164.1 (C=O), 166.1 (C=O). **MS** (ESI⁺) m/z 335.0 [M+H]⁺. Anal. calcd. for C₁₅H₁₄N₂O₅S (334.35): C, 53.89; H, 4.22; N, 8.38. Found: C, 53.72; H, 4.33; N, 8.18 %.

4.1.5.2. 2-((Z)-5-(3,4,5-Trimethoxybenzylidene)thiazolidine-2,4-dione-3-yl)acetamide (8)

White crystals (recrystallization from ethanol: dioxane mixture (1:3)), yield 0.26 g (83%), mp 274-275 °C (decomposition). **IR** (ν cm^{-1}): 3384, 3304 and 3264 (NH₂) 3026 (CH arom), 2974 (CH aliph.), 2839 (CH aliph.), 1740 (C=O), 1682 (C=O), 1604, 1579, 1508 and 1448 (C=C arom.). **$^1\text{H-NMR}$** (300 MHz, $\text{DMSO-}d_6$) δ (ppm): 3.70 (s, 3H, -OCH₃), 3.85 (s, 6H, 2 -OCH₃), 4.24 (s, 2H, CH₂), 6.97 (s, 2H, ArH), 7.32 (s, 1H, NH), 7.73 (s, 1H, NH), 7.92 (s, 1H, =CH-). **$^{13}\text{C-NMR}$** (75 MHz, $\text{DMSO-}d_6$) δ (ppm): 43.8 (N-CH₂), 56.5 (2 -OCH₃), 60.7 (-OCH₃), 108.1 (2 CH), 120.6 (C), 128.9 (C), 133.9 (C), 140.1 (CH), 153.7 (2 C),

165.7 (C=O), 167.3 (C=O), 167.5 (C=O). MS (ESI⁺) m/z 353.0 [M+H]⁺. Anal. calcd. for C₁₅H₁₆N₂O₆S (352.36): C, 51.13; H, 4.58; N, 7.95. Found: C, 50.89; H, 4.60; N, 7.78 %.

4.1.5.3. Ethyl ((Z)-5-(3,4,5-trimethoxybenzylidene)thiazolidine-2,4-dion-3-yl)acetate (9)

White crystals (recrystallization from ethanol), yield 0.22 g (64%), mp 145-147 °C. **IR** (v cm⁻¹): 3018 (CH arom.), 2999 (CH aliph.), 2942 (CH aliph.), 2842 (CH aliph.), 1760 (C=O), 1739 (C=O), 1689 (C=O), 1602, 1575, 1504 and 1469 (C=C arom.). **¹H-NMR** (300 MHz, DMSO-*d*₆) δ (ppm): 1.22 (t, 3H, *J* = 7.1 Hz, OCH₂CH₃), 3.74 (s, 3H, -OCH₃), 3.84 (s, 6H, 2 -OCH₃), 4.18 (q, 2H, *J* = 7.1 Hz, OCH₂CH₃), 4.50 (s, 2H, CH₂), 6.98 (s, 2H, ArH), 7.95 (s, 1H, =CH-). **¹³C-NMR** (75 MHz, DMSO-*d*₆) δ (ppm): 14.4 (CH₃), 42.7 (-NCH₂), 56.5 (2 -OCH₃), 60.7 (-OCH₃), 62.1 (OCH₂), 108.2 (2 CH), 119.9 (C), 128.7 (C), 134.8 (C), 140.3 (CH), 153.7 (2C), 165.3 (C=O), 167.1 (C=O), 167.3 (C=O). MS (ESI⁺) m/z 382.0 [M+H]⁺. Anal. calcd. for C₁₇H₁₉NO₇S (381.40): C, 53.54; H, 5.02; N, 3.67. Found: 53.38; H, 5.15; N, 3.47 %.

4.1.5.4. (Z)-3-(2-Oxopropyl)-5-(3,4,5-trimethoxybenzylidene)thiazolidine-2,4-dione (10)

Yellow crystals (recrystallization from ethanol), yield 0.152 g (82%), mp 139-140 °C. **IR** (v cm⁻¹): 3026 (CH arom.), 2939 (CH aliph.), 2839 (CH aliph.), 1748 (C=O), 1726 (C=O), 1691 (C=O), 1604, 1576, 1506 and 1456 (C=C arom.). **¹H-NMR** (300 MHz, DMSO-*d*₆) δ (ppm): 2.25 (s, 3H, CH₃), 3.74 (s, 3H, -OCH₃), 3.84 (s, 6H, 2 -OCH₃), 4.67 (s, 2H, CH₂), 6.98 (s, 2H, ArH), 7.92 (s, 1H, =CH-). **¹³C-NMR** (75 MHz, DMSO-*d*₆) δ (ppm): 27.5 (CH₃), 50.8 (-NCH₂), 56.3 (2 -OCH₃), 60.7 (-OCH₃), 108.2 (2 CH), 120.3 (C), 128.8 (C), 134.4 (C), 140.1 (CH), 153.7 (2 C), 165.5 (C=O), 167.3 (C=O), 200.8 (C=O). MS (ESI⁺)

m/z 352.0 [M+H]⁺. Anal. calcd. for C₁₆H₁₇NO₆S (351.37): C, 54.69; H, 4.88; N, 3.99. Found: C, 54.48; H, 4.97; N, 3.71 %.

4.1.6. General procedure for the synthesis of compounds 11-16.

A mixture of the potassium salt **3** (0.33 g, 1 mmol) and dichloroethane (0.098 g, 0.07 ml, 1 mmol), 1,3-dichloropropane (0.113 g, 0.1 ml, 1 mmol), 1,4-dibromobutane (0.216 g, 0.118 ml, 1 mmol), 1,5-dibromopentane (0.23 g, 0.136 ml, 1 mmol), 1,6-dibromohexane (0.244 g, 0.153 ml, 1 mmol) or 1,8-dibromooctane (0.272 g, 0.184 ml, 1 mmol) in DMF (5 ml) was heated under reflux for 6 hr. After cooling the solid product obtained was filtered, washed with water, dried and recrystallized.

4.1.6.1. 1,2-Bis(5-Z-(3,4,5-trimethoxybenzylidene)thiazolidin-2,4-dione-3-yl)ethane (11)

White crystals (recrystallization from ethanol), yield 0.49 g (88%), mp 237-238 °C. IR (v cm⁻¹): 3018 (CH arom.), 2931 (CH aliph.), 2825 (CH aliph.), 1739 (C=O), 1678 (C=O), 1607, 1577, 1507 and 1465 (C=C arom.). ¹H-NMR (300 MHz, DMSO-*d*₆) δ (ppm): 3.72 (s, 6H, 2 -OCH₃), 3.82 (s, 12H, 4 -OCH₃), 3.93 (s, 4H, -N(CH₂)₂N-), 6.93 (s, 4H, 2 ArH), 7.87 (s, 2H, 2 =CH-). ¹³C-NMR (75 MHz, DMSO-*d*₆) δ (ppm): 48.6 (2 -NCH₂), 56.5 (4 -OCH₃), 60.7 (OCH₃), 66.8 (OCH₃), 108.2 (4 CH), 120.1 (2 C), 128.7 (2 C), 134.6 (2 C), 140.2 (2 CH), 153.7 (4 C), 165.3 (2 C=O), 167.2 (2 C=O). MS (ESI⁺) m/z 617.1 [M+H]⁺. Anal. calcd for C₂₈H₂₈N₂O₁₀S₂ (616.66): C, 54.54; H, 4.58; N, 4.54. Found: C, 54.32; H, 4.78; N, 4.50 %.

4.1.6.2. 1,3-Bis(5-Z-(3,4,5-trimethoxybenzylidene)thiazolidin-2,4-dione-3-yl)propane (12)

Yellow crystals (recrystallization from ethanol), yield 0.223 g (39%), mp 174-176 °C. IR (v cm⁻¹): 3037 (CH arom.), 2931 (CH aliph.), 2837(CH aliph.), 1735 (C=O), 1678 (C=O),

1607, 1577, 1507 and 1454 (C=C arom.). **¹H-NMR** (300MHz, DMSO-*d*₆) δ (ppm): 2.01 (m, 2H, *J* = 6.5 Hz, CH₂), 3.72 (m, 10H, 2 -OCH₃ and 2 NCH₂), 3.81 (s, 12H, 4 -OCH₃), 6.9 (s, 4H, ArH), 7.85 (s, 2H, 2 =CH). **¹³C-NMR** (75 MHz, DMSO-*d*₆) δ (ppm): 25.2 (CH₂), 41.8 (2-NCH₂), 56.4 (4 OCH₃), 60.6 (2 OCH₃), 108.1 (4 CH), 120.5 (2 C), 128.8 (2 C), 133.6 (2 C), 140.0 (2 CH), 153.6 (4 C), 166.0 (2 C=O), 167.7 (2 C=O). MS (ESI⁺) *m/z* 631.1 [M+H]⁺. Anal. calcd for C₂₉H₃₀N₂O₁₀S₂ (630.68): C, 55.23; H, 4.79; N, 4.44. Found: 55.00; H, 4.98; N, 4.31 %.

4.1.6.3. 1,4-bis(5-Z-(3,4,5-trimethoxybenzylidene)thiazolidin-2,4-dione-3-yl)butane (13)

White crystals (recrystallization from ethanol), yield 0.205 g (71%), mp 207-208 °C. **IR** (ν cm⁻¹): 3020 (CH arom.) 2936 (CH aliph.), 2836 (CH aliph.), 2835 (CH aliph.), 1742 (C=O), 1678 (C=O), 1608, 1579, 1506 and 1463 (C=C arom.). **¹H-NMR** (300MHz, DMSO-*d*₆) δ (ppm): 1.60 (m, 4H, CH₂-CH₂), 3.66 (m, 4H, 2 NCH₂), 3.72 (s, 6H, 2 -OCH₃), 3.82 (s, 12H, 4 -OCH₃), 6.92 (s, 2H, ArH), 7.86 (s, 2H, 2 =CH). **¹³C-NMR** (75MHz, DMSO-*d*₆) δ (ppm): 24.8 (2 CH₂), 41.5 (2 -NCH₂), 56.5 (4 -OCH₃), 60.7 (2 -OCH₃), 108.1 (4 CH), 120.7 (2 C), 128.9 (2 C), 133.6 (2 C), 140.0 (2 CH), 153.7 (4 C), 166.1 (2 C=O), 167.9 (2 C=O). MS (ESI⁺) *m/z* 645.1 [M+H]⁺. Anal. calcd. for C₃₀H₃₂N₂O₁₀S₂ (644.71): C, 55.89; H, 5.00; N, 4.35. Found: C, 55.66; H, 5.17; N, 4.16 %.

4.1.6.4. 1,5-Bis(5-Z-(3,4,5-trimethoxybenzylidene)thiazolidin-2,4-dione-3-yl)pentane. (14)

Off-white crystals (recrystallization from ethanol:dioxane mixture (1:3)), yield 0.212 g (75%), mp 147-148 °C. **IR** (ν cm⁻¹): 3014 (CH arom.), 2943 (CH aliph.), 2825 (CH aliph.), 1742 (C=O), 1678 (C=O), 1608, 1579, 1506 and 1463 (C=C arom.). **¹H-NMR** (300MHz, DMSO-*d*₆) δ (ppm): 1.20 (m, 2H, CH₂), 1.60 (m, 4H, 2 CH₂), 3.60 (t, 4H, *J* = 4.6 Hz, 2 -

NCH₂), 3.72 (s, 6 H, 2 -OCH₃), 3.80 (s, 12 H, 4 -OCH₃), 6.90 (s, 4H, ArH), 7.84 (s, 2H, 2 =CH-). ¹³C-NMR (75 MHz, DMSO-*d*₆) δ (ppm): 23.7 (CH₂), 27.0 (2 CH₂), 41.6 (2 N-CH₂), 56.4 (4 OCH₃), 60.6 (2 OCH₃), 108.0 (4 CH), 120.7 (2 C), 128.9 (2 C), 133.6 (2 C), 139.9 (2 CH), 153.6 (4 C), 166.1 (2 C=O), 167.8 (2 C=O). MS (ESI⁺) *m/z* 659.1 [M+H]⁺. Anal. calcd for C₃₁H₃₄ N₂O₁₀S₂ (658.74). C, 56.52; H, 5.20; N, 4.25. Found: C, 56.42; H, 5.35; N, 4.06 %.

4.1.6.5. 1,6-Bis(5-Z-(3,4,5-trimethoxybenzylidene)thiazolidin-2,4-dione-3-yl)hexane (15)

Yellow needles (recrystallization from ethanol-dioxane mixture (1:3)), yield 0.25 g (80%), mp 203-205 °C. IR (ν cm⁻¹): 3017 (CH arom.), 2941 (CH aliph.), 2836 (CH aliph.), 1740 (C=O), 1676 (C=O), 1609, 1577, 1507 and 1454 (C=C arom.). ¹H-NMR (300 MHz, CDCl₃) δ (ppm): 1.40 (m, 4H, 2 CH₂), 1.70 (m, 4H, 2 CH₂), 3.76 (t, 4H, *J* = 7.2 Hz, 2 -NCH₂), 3.92 (s, 18H, 6 -OCH₃), 6.75 (s, 4H, ArH), 7.82 (s, 2H, 2 =CH-). ¹³C-NMR (75 MHz, CDCl₃) δ (ppm): 26.2 (2 CH₂), 27.6 (2 CH₂), 41.8 (2 -NCH₂), 56.2 (4 -OCH₃), 61.0 (2 -OCH₃), 107.5 (4 CH), 120.5 (2 C), 128.7 (2 C), 133.8 (2 C), 140.2 (2 CH), 153.6 (4 C), 166.3 (2 C=O), 167.8 (2 C=O). MS (ESI⁺) *m/z* 673.2 [M+H]⁺. Anal. calcd. for C₃₂H₃₆ N₂O₁₀S₂ (672.76): C, 57.13; H, 5.39; N, 4.16. Found: C, 56.87; H, 5.47; N, 3.99 %.

4.1.6.6. 1,8-Bis(5-Z-(3,4,5-trimethoxybenzylidene)thiazolidin-2,4-dione-3-yl)octane (16)

A mixture of the potassium salt **3** (0.66 g, 2 mmol) and 1,8-dibromooctane (0.272 g, 0.184 ml, 1 mmol) in DMF (5 ml) was heated under reflux for 6 hr. After cooling the solid product obtained was filtered, washed with water and dried. Recrystallization from dioxane gave yellow needles, yield 0.212 g (71%), mp 168-170 °C. IR (ν cm⁻¹): 3015 (CH arom.), 2939

(CH aliph.), 2854 (CH aliph.), 1742 (C=O), 1679 (C=O), 1607, 1576, 1507 and 1453 (C=C arom.). **¹H-NMR** (300 MHz, CDCl₃) δ (ppm): 1.35 (m, 8H, 4 CH₂), 1.65 (m, 4H, 2 CH₂), 3.75 (t, 4H, *J* = 7.15 Hz, 2 -NCH₂), 3.92 (s, 18H, 6 -OCH₃), 6.75 (s, 4H, ArH), 7.84 (s, 2H, 2 =CH-). **¹³C-NMR** (75 MHz, CDCl₃) δ (ppm): 26.6 (2 CH₂), 27.7 (2 CH₂), 28.9 (2 CH₂), 42.0 (2 -NCH₂), 56.2 (4 -OCH₃), 61.0 (2 -OCH₃), 107.5 (4 CH), 120.6 (2 C), 128.7 (2 C), 133.7 (2 C), 140.2 (2 CH), 153.6 (4 C), 166.3 (2 C=O), 167.8 (2 C=O). MS (ESI⁺) *m/z* 701.2 [M+H]⁺. Anal. calcd. For C₃₄H₄₀ N₂O₁₀S₂ (700.82): C, 58.27; H, 5.75; N, 4.00. Found: C, 58.00; H, 5.83; N, 3.88 %.

4. 2. Biological assays

4. 2. 1. Cell culture and reagents

Human MDA-MB-231 and MCF-7 breast cancer cells were obtained from the Vacsera (Giza, Egypt), and were maintained in a culture medium consisting of MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS, EuroClone, Life Science Division, Milan, Italy) as previously described [43]. The cell cultures were free of *Mycoplasma*, as assessed using an enzyme-linked immunosorbent assay (ELISA) kit (Boehringer, Mannheim, Germany). The anti-proliferative effect of the three thiazolidinediones **5**, **7** and **9** was evaluated on these cell lines as well as on the human breast cancer cells that were isolated from patients with breast cancer was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake method as previously described [44]. The cells were plated at 1×10^6 cells/ml in 2 ml of culture medium in six-well Costar plates (Corning, Corning, NY). The cells were treated with different concentrations of **5**, **7** and **9** for 12 h, and cytotoxicity was expressed as a relative percentage of the OD values measured

in the control (medium) and chemical compounds-treated cells. Morphological changes were observed after exposure to medium and these chemical compounds using a phase-contrast inverse microscope (Olympus, Japan).

4.2.2. Isolation of human normal non-cancerous breast cells and human breast cancer cells.

Human breast cancer cells and human normal non-cancerous breast cells were obtained from the same three female patients at the time of surgery after informed written consent was obtained. Patients were treated at South Egypt Cancer Institute, Assiut University, Egypt.

Patients	Age	Patient History
Patient 1	46	New case
Patient 2	64	Subjected to a surgery and received chemotherapy and radiotherapy.
Patient 3	53	New case

4.2.3. Apoptosis detection and flow cytometry analysis

The percentage of breast cancer cells undergoing apoptosis was determined by flow cytometry after 12 hours of incubation with medium or **5**, **7** and **9**. Dead cells were identified using the Trypan blue exclusion test. However, to distinguish between viable, early apoptotic and late apoptotic cells, the human breast cancer cells and human normal non-cancerous breast cells were washed and incubated in PBS containing 30% human AB serum (4°C for 30 minutes) prior to staining with Annexin V-FITC and propidium iodide (PI) (15 minutes at 25°C) using a commercial kit according to the manufacturer's instructions (Abcam, Canada). The cells were analyzed by flow cytometry using a

FACSCalibur flow cytometer (BD-Pharmingen) within 1 hour of staining, and the percentage of cells undergoing apoptosis was determined as previously described [45-47].

4.2.4. Western blot analyses

Western blot analysis was performed as previously described [46, 47]. Briefly, whole-cell lysates were prepared from human breast cancer cells and human normal non-cancerous breast cells which were isolated from the same breast cancer patients, as well as from human breast cancer cell lines (MDA-MB-231 and MCF-7). Cells were firstly treated for 12 h with medium (0) or the three TZDs **5**, **7** and **9** in RIPA buffer (20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1.0% Triton X100, 0.1% SDS, 1% sodium deoxycholate, 10% glycerol, 1 mM EDTA and 1% protease inhibitor cocktail, Roche). Following centrifugation at $16,000 \times g$ at 4°C for 15 min, the protein concentrations in the supernatants were determined using a protein assay kit (Bio-Rad, Hercules, CA) and BCA protein assay method. Equal amounts of whole-cell protein (50 µg) were mixed with reducing sample buffer (0.92 M Tris-HCl, pH 8.8, 1.5% SDS, 4% glycerol, and 280 mM 2-Mercaptoethanol) and separated using discontinuous SDS-PAGE. Proteins were transferred onto nitrocellulose membranes using a Bio-Rad Trans-Blot electrophoretic transfer device, and the membranes were blocked for 1 h at room temperature with 1% BSA or 5% skim milk dissolved in TBS (20 mM Tris-HCl, pH 7.4, and 150 mM NaCl) supplemented with 0.1% Tween 20. The membranes were then incubated in the same blocking buffer with anti-phospho-AKT (p-AKT), anti-active total-AKT (T-AKT), anti-phospho-mTOR (p-mTOR), anti-total-mTOR (T-mTOR), anti-VEGF, anti-HIF-1 α , anti- β -actin, anti-Bcl-2, anti-Bcl-XL, anti-Mcl-1, anti-Bak, anti-Bax, anti-Bim or anti-GAPDH antibodies (all from Cell Signaling Technology, Beverly, MA). The blots were thoroughly rinsed and then incubated

with an HRP-labeled species-matched secondary antibody for another 1 h. Protein bands were detected using enhanced chemiluminescence reagents (ECL, SuperSignal West Pico Chemiluminescent Substrate, Perbio, Bezons, France), and the ECL signals were recorded on Hyperfilm ECL. To quantify band intensities, the films were scanned, saved as TIFF files and analyzed using NIH ImageJ software.

4.2.5. Statistical analyses

Data were first tested for normality (using Anderson–Darling test) and for variances homogeneity prior to any further statistical analysis. Data were normally distributed and were expressed as the mean \pm standard error of the mean (SEM). Significant differences among groups were analyzed using a one-way analysis of variance using SPSS software, version 17. Differences were considered statistically significant at $P < 0.05$. * $P < 0.05$, **5**-, **7**- and **9**-treated cells vs. medium-treated cells.

Conflict of interest:

The authors declare no conflicts of interest. This manuscript has not been published or submitted elsewhere. This work complies with the Ethical Policies of this Journal and has been conducted under internationally accepted ethical standards after relevant ethical review. All authors have approved the final article.

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Appendix A. Supplementary data

IR, NMR and MS spectra are available as supplementary data.

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Figure legends

Figure 1. Dose-dependent responses of cell viability after treatment with medium, 5, 7 and 9.

Human breast cancer cells that were isolated from human breast cancer tissues were firstly treated for 12 hours with medium (0), 5, 7 and 9 at different concentrations. The experiments were performed in triplicate, and the results are expressed as the mean percentage of viable cells \pm SEM. * $P < 0.05$, 5-, 7- or 9-treated cells vs. medium-treated cells (n=3).

Figure 2. Influence of 5, 7 and 9 on the induction of apoptosis in breast cancer cells

The potential of these three TZDs to induce apoptosis was evaluated using annexin V/PI assay followed by flow cytometry analysis. The analysis of annexin V/PI double stained cells was performed after gating on the homogenous cell population. (A) One representative experiment of the three is shown for dot plots of annexin V and PI stained human normal non-cancerous and breast cancer cells. Data from three different experiments are expressed as the mean percentage of apoptotic cells \pm SEM for the medium (untreated, open bars)-, **7**- (closed black bars) (B), **9**- (closed gray bars) (C) cells and **5**- (hatched bars) (D) cells. *P < 0.05, **5**-, **7**- or **9**-treated cells vs. medium-treated cells (n=3).

Figure 3. Effect of 5, 7 and 9 on the phosphorylation of AKT and mTOR, and on the expression of VEGF and HIF-1 α in MCF-7 cells.

The phosphorylation of AKT and mTOR and the expression of VEGF and HIF-1 α were monitored in MCF-7 cells using western blot analysis. Protein bands from one representative experiment of the five performed are shown for the phosphorylation of AKT (A) and mTOR (C) and the expression of VEGF and HIF-1 α and β -actin (E) in MCF-7 cells. The phosphorylation of AKT and mTOR was normalized to the total relevant protein levels T-AKT and T-mTOR respectively. The expression of VEGF and HIF-1 α was normalized to the total β -actin levels. The results are expressed as the mean \pm SEM of normalized AKT (B), mTOR (D) and VEGF and HIF-1 α (F) in the medium (0)- (open bars), **7**- (closed black bars), **9**- (closed gray bars) and **5**-treated (hatched bars) MCF-7 cells. *P < 0.05, **5**-, **7**- or **9**-treated cells vs. medium-treated cells (n=5).

Figure 4. The impact of 5, 7 and 9 on the phosphorylation of AKT and mTOR, and on the expression of VEGF and HIF-1 α in human normal non-cancerous breast cells and breast cancer cells.

Medium (0)-, **5**-, **7**- and **9**- treated human normal non-cancerous breast cells and breast cancer cells for 12 h were lysed and subjected to Western blotting using antibodies recognizing p-AKT, T-AKT, p-mTOR, T-mTOR, VEGF, HIF-1 α and β -actin. Protein bands from one representative experiment of the five performed are shown for the phosphorylation of AKT (**A**) and mTOR (**C**) and the expression of VEGF and HIF-1 α and β -actin (**E**) in the human normal breast cells and breast cancer cells. The phosphorylation of AKT and mTOR was normalized to the total relevant protein levels T-AKT and T-mTOR respectively. The expression of VEGF and HIF-1 α was normalized to the total β -actin levels. The results are expressed as the mean \pm SEM of normalized AKT (**B**), mTOR (**D**) and VEGF and HIF-1 α (**F**) in the medium (0)- (open bars), **7**- (closed black bars), **9**- (closed gray bars) and **5**-treated (hatched bars) human normal non-cancerous breast cells and breast cancer cells. *P < 0.05, **5**-, **7**- or **9**-treated cells vs. medium-treated cells (n=5).

Figure 5. The effect of 5, 7 and 9 on the expression of Bcl-2 family members in MCF-7 cells.

Medium (0)-, **5**-, **7**- and **9**-treated MCF-7 cells for 12 h were lysed and subjected to Western blotting using antibodies recognizing Bcl-2, Bcl-XL, Mcl-1, Bak, Bax, Bim and GAPDH. Protein bands from one representative experiment of the five performed are shown for the expression of the anti-apoptotic Bcl-2 family members (**A**) and pro-apoptotic Bcl-2 family members (**B**) in MCF-7 cells. The expression of Bcl-2, Bcl-XL, Mcl-1, Bak, Bax and Bim was normalized to the total GAPDH levels. The results are expressed as the mean \pm SEM of normalized anti-apoptotic Bcl-2 family members (**C**) and pro-apoptotic Bcl-2 family members (**D**) in the medium (0)- (open bars), **7**- (closed black bars), **9**- (closed gray bars)

and **5**-treated (hatched bars) MCF-7 cells. *P < 0.05, **5**-, **7**- or **9**-treated cells vs. medium-treated cells (n=5).

Figure 6. The effect of 5, 7 and 9 on the expression of Bcl-2 family members in MDA-MB-231 cells.

Medium (0)-, **5**-, **7**- and **9**-treated MDA-MB-231 cells for 12 h were lysed and subjected to Western blotting using antibodies recognizing Bcl-2, Bcl-XL, Mcl-1, Bak, Bax, Bim and GAPDH. Protein bands from one representative experiment of the five performed are shown for the expression of the anti-apoptotic Bcl-2 family members (**A**) and pro-apoptotic Bcl-2 family members (**B**) in MCF-7 cells. The expression of Bcl-2, Bcl-XL, Mcl-1, Bak, Bax and Bim was normalized to the total GAPDH levels. The results are expressed as the mean \pm SEM of normalized anti-apoptotic Bcl-2 family members (**C**) and pro-apoptotic Bcl-2 family members (**D**) in the medium (0)- (open bars), **7**- (closed black bars), **9**- (closed gray bars) and **5**-treated (hatched bars) MDA-MB-231 cells. *P < 0.05, **5**-, **7**- or **9**-treated cells vs. medium-treated cells (n=5).

Figure 7. The effect of 5, 7 and 9 on the expression of Bcl-2 family members in human normal non-cancerous breast cells and breast cancer cells.

Medium (0)-, **5**-, **7**- and **9**-treated human normal non-cancerous breast cells and breast cancer cells for 12 h were lysed and subjected to Western blotting using antibodies recognizing Bcl-2, Bcl-XL, Mcl-1, Bak, Bax, Bim and GAPDH. Protein bands from one representative experiment of the five performed are shown for the expression of the anti-apoptotic Bcl-2 family members (**A**) and pro-apoptotic Bcl-2 family members (**B**) in the human normal non-cancerous breast cells and breast cancer cells. The expression of Bcl-2, Bcl-XL, Mcl-1, Bak, Bax and Bim was normalized to the total GAPDH levels. The results

are expressed as the mean \pm SEM of normalized anti-apoptotic Bcl-2 family members (**C**) and pro-apoptotic Bcl-2 family members (**D**) in the medium (0)- (open bars), **7**- (closed black bars), **9**- (closed gray bars) and **5**-treated (hatched bars) cells. * $P < 0.05$, **5**-, **7**- or **9**-treated cells vs. medium-treated cells (n=5).